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MIPR NO. 94MM4529

TITLE: Phytoestrogens in the Food Supply

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CONTRACTING ORGANIZATION: U.S. Department of Agriculture
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REPORT DATE: January 1996

TYPE OF REPORT: Midterm

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
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1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE January 1996	3. REPORT TYPE AND DATES COVERED Midterm (15 Mar 94 - 31 Dec 95)	
4. TITLE AND SUBTITLE Phytoestrogens in the Food Supply			5. FUNDING NUMBERS 94MM4529	
6. AUTHOR(S) Gary R. Beecher, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) U.S. Department of Agriculture Beltsville, Maryland 20705-2350			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Commander U.S. Army Medical Research and Materiel Command Fort Detrick Frederick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) Endogenous estrogens are thought to have a role in the etiology of breast cancer. Several components of plant foods have estrogenic activity or are metabolized to active compounds by mammalian systems (phytoestrogens). The impact of these constituents on breast cancer risk is unknown because of the lack of databases containing appropriate values for foods. The goal of this research is to provide a database of values for the various isoflavones (one family of phytoestrogens) in soy-based foods and commodities. Six critical research components have been identified: 1) develop and validate rugged analytical procedures, 2) establish appropriate analytical quality control system, 3) identify commonly consumed soy-based foods and commodities, 4) develop food sampling scheme for each food or commodity, 5) prepare and analyze foods, and 6) develop critical data evaluation system and prepare database of values. Research components one through three have been completed. Several foods have been sampled, based on appropriate sampling schemes, prepared and analyzed. Additional foods will be sampled and analyzed as food sales information is received. The data evaluation system currently is being modified to accept and review isoflavone data. When all data have been entered into this system, a database of isoflavone values complete with food names and codes will be produced.				
14. SUBJECT TERMS breast cancer			15. NUMBER OF PAGES 25	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

19960719 155

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E. Introduction

E.1. Nature of Problem

Endogenous estrogens are thought to play a role in the etiology of breast cancer. Phytoestrogens are important constituents of selected foods (primarily soybean products) whose metabolite(s) may interact with estrogen receptor sites thereby modulating the response of cells and tissues to estrogen (anti-estrogen activity). The impact of these food constituents on breast cancer risk and prevention is unknown at this time but can be ascertained with an accurate and reliable database of analytical values for foods. At this time, such a database is not available. The results of the proposed research will generate such a database which will allow the importance of the food sources of natural "anti-estrogens" to be critically evaluated.

E.2. Background

E.2.1. Estrogen as an important risk factor for cancer. Several reports have suggested an important role for endogenous estrogens in the development of cancers of the reproductive tract (1). Estrogens are hypothesized to play a role as carcinogens or co-carcinogens in the tumorigenic process as well as in the growth of established tumors (2). The risk factor of significance appears to be cumulative lifetime estrogen exposure. As a result of the significant role of estrogens in carcinogenesis, synthetic anti-estrogens are routinely given to treat estrogen-dependent breast carcinoma (3).

In addition to estradiol, the primary circulating estrogen in premenopausal women, it is necessary also to consider exposure to estrone, the primary circulating estrogen in postmenopausal women, which originates from extraglandular conversion of adrenal androstenedione (4). Breast cancer patients have been shown to have higher levels of estrone-sulfate than controls (5). That adipose tissue is the primary site of the conversion may explain the increased risk of endometrial carcinoma in obese postmenopausal women (6).

Hepatic estrogen metabolism and conjugation also has been shown to be important in regard to cancer incidence. Breast and endometrial cancer patients show increased estrogen metabolism via the 16-hydroxylation pathway to 16-alpha-hydroxyestrone and estriol, compounds with demonstrable estrogenic activity, as opposed to the inert estrogens formed by 2-hydroxylation (7). In addition, breast cancer patients have been reported to have higher circulating levels of estrogen sulfates and lower levels of estrogen glucuronides (8).

E.2.2. Association of diet with altered estrogen levels. Many studies report that diet affects the production and excretion of endogenous estrogens in humans. Vegetarian diet results in anovulation and decreased luteal phase estradiol levels in healthy young women (9). Vegetarian women have lowered urinary (10) and plasma (11,12) estrogen levels and increased fecal (11) estrogens. Increased sex hormone binding globulin (SHBG), which lowers free estradiol levels, also has been reported in vegetarians (10).

Low-fat, high fiber diets have been shown to influence hepatic estrogen sulfation and metabolism. Woods, et al., reported decreased circulating estrone-sulfate levels in women on a low-fat, high fiber diet (13). Longcope, et al., reported decreased urinary excretion of the active 16-alpha-hydroxylated estrogen metabolites, estriol and 16-alpha-hydroxyestrone, and increased excretion

of the inactive 2-alpha-hydroxylated estrogens, in women on a low-fat diet (14).

It is very difficult to determine the nutrients and other dietary components responsible for the estrogen-lowering effects of a vegetarian, or low fat, high fiber diet. In general, the reported studies do not differentiate the effects of lowered dietary total fat, saturated fat and cholesterol; increased dietary fiber; and increased legume, grain, fruit and vegetable consumption. If dietary recommendations for cancer-prevention are to be developed, it is extremely important to identify the specific dietary substances of significance.

E.2.3. Association of phytoestrogens with altered estrogen levels and action. One of the most important classes of dietary compounds known to influence estrogen levels and action are the phytoestrogens, plant compounds that possess weak estrogenic activity. The earliest indications of the physiological significance of phytoestrogens were reports of Clover Disease, an infertility syndrome in sheep grazing on phytoestrogen-rich clover (15). The phytoestrogens in clover were found to be isoflavones, heterocyclic phenols similar in structure to natural and synthetic estrogens. The isoflavones of greatest abundance in the food supply are daidzein, genistein, and glycitein and their glucosidic derivatives (16, 17). Equol is an isoflavone metabolite of daidzein (4-deoxy daidzein), produced by gut bacteria, which is thought to be physiologically active.

Isoflavones have been shown to possess a variety of estrogenic activities, both *in vivo* and *in vitro*. Animal studies have shown diminished fertility in a variety of species consuming phytoestrogen-rich clover, as a result of the estrogenic actions of daidzein and equol produced from the high levels of formononetin present in the clover (16). In these studies, defections have been found in the hypothalamic-pituitary-gonadal regulation of estrus. Postmenopausal women consuming isoflavones have exhibited an estrogenic response, as reflected in increased growth of the vaginal epithelium (18). *In vitro* studies have shown daidzein, equol and genistein to bind estrogen receptors (19-21).

Under certain circumstances, isoflavones have been shown to act as anti-estrogens. As weak estrogens competing for the estrogen receptor, phytoestrogens may act as "natural" anti-estrogens by blocking the receptor binding of potent estrogens. For example, equol has been shown to block the uterotrophic effects of estradiol when they are injected simultaneously in rats (20). Isoflavones also have been shown to increase SHBG levels, resulting in decreased free estradiol levels (22). Another mechanism for anti-estrogenic action of isoflavones was revealed by recent studies which showed Biochanin A (4'-methoxy genistein) to inhibit estrogen synthesis in preadipocytes (23).

E.2.4. Association of phytoestrogens (isoflavones) with decreased breast cancer. The anti-estrogenic effects of isoflavones have been suggested to contribute to the decreased cancer risk observed in populations consuming foods rich in phytoestrogens. Consumption of soybeans has been suggested to contribute to the low incidence of breast and prostate cancer observed in Japanese women and men (24) and dietary soy has been shown to be inversely associated with breast cancer risk in Singapore (25). In the Japanese women, an average consumption of 54g/day of soy products plus about 20g/day of soy sauce resulted in urinary excretion of total isoflavones (daidzein, equol, O-desmethylangolensin) of 7 μ moles/day. These women excreted 10 fold more daidzein and 20-30 fold more equol and O-desmethylangolensin than omnivorous and lactovegetarian women residing in Helsinki and Boston (24). In one of few controlled soy feeding studies that has been reported in humans, equol excretion increased by 1000-fold in four (two women and two men) out of six subjects, within one day of consumption of 40 g dry weight/day of cooked textured soy (26). It is interesting to note that equol was increased to 1000 times the level of estrone-glucuronide, the principal urinary estrogen, during the follicular phase of the menstrual cycle (26).

In animals, soy consumption results in uterotrophic effects thought to be caused by the isoflavone content (27). High consumption of soy also has resulted in infertility diseases in various species, similar to the pathology of Clover Disease (16). Animal studies have shown that dietary soy decreases mammary tumor development in rats (28) and reduces precancerous changes in a mouse prostatic cancer model (29). Miso (30) and powdered soybean chips (31) both before and after protease inhibitor denaturation decrease mammary tumor development in rats. Cell studies show that flavonoid compounds inhibit growth of the human breast cancer cell line ZR-75-1 (32).

E.2.5. Other anticancer activities of phytoestrogens (isoflavones). Isoflavones have been shown to possess several additional biological activities that may be important relative to reducing the risk of cancer. Naim et al., reported that isoflavones inhibited lipoxygenase action and prevented peroxidative hemolysis of sheep erythrocytes in vitro (33). The extent depended on the structure of the isoflavones. Pratt and Birac found that soybeans, defatted soy flour, soy protein concentrates, and soy isolates had appreciable antioxidant activities detected by the rate of β -carotene bleaching in a lipid-aqueous system, which was due to phenolic compounds (34). Presumably the active components in these preparation were isoflavones. Malonyl isoflavones functioned as good antioxidants in the storage test carried out at 37°C and in UV-light induced oxidation of the β -carotene/linoleic acid system (35).

In vitro, genistein specifically inhibits epidermal growth factor receptor tyrosine kinase activity and protein histidine kinase from yeast cell extracts (36,37). Genistein is a unique topoisomerase inhibitor in selectively suppressing the growth of oncogene *ras*-transformed NIH 3T3 cells but not normal NIH 3T3 cells (38). In addition, Sariasiani and Kunz (39) found soybean flour and genistein induced cytochrome P-450 in *Streptomyces griseus*. In a short-term rat study, hepatic cumene hydroperoxidase activity was increased by feeding a soy isoflavone extract (40). These findings may be alternative mechanisms of inhibition of nonhormone-related tumorigenesis by soy isoflavones.

E.2.6. Dietary sources of phytoestrogens (isoflavones). Soybeans, soybean products and other legumes are excellent sources of isoflavones. Walter is credited with the first report of isoflavones in foods (41). Murphy reported the isoflavone content of eight different soy food types (42). Murphy et al. (43) and Eldridge (44) reported isoflavone contents of soy isolates concentrates and flours. The isoflavone content of selected soy protein preparations also has been reported (45).

Farmakalidis and Murphy (46) reported two new isoflavonoid species that appeared to be the result of heat processing, 6"-O-acetyl-genistin and -daidzin. These new isoflavones were characterized by melting point, uv, infrared, proton-NMR and mass spectrometry, and elemental analysis. These characteristics were comparable with those reported by Ohta et al. (47, 48). The amounts of these isoflavonoids and their parent compounds were reported for eight soybean varieties and conditions. Two reports (49, 50) have examined the isoflavone content of foods by measuring the genistein and daidzein present before and after acid hydrolysis (i.e., by difference), thus, missing any unique isoflavonoid forms similar to those reported by Farmakalidis and Murphy (51). Graham (52) recently reported a 6"-O-malonyl-genistin and -daidzin in soy flours. The compounds were reported to be identified by mass spectrometry but no spectra were published.

Two additional isoflavones from soybeans have been reported; one of them has been confirmed recently by Murphy and her colleagues. Glycitein (6-methoxy, 4',7-dihydroxyisoflavone), reported by Naim et al. (33), was isolated from a concentrated syrup extract of soy flakes. This extract was prepared by long heating to reduce the volume. As a result, glycitein may be an artifact of sample

processing. Gyorgy et al. (53) reported the presence of 6,7,4'-trihydroxyisoflavone in tempeh, a soybean product made by fermentation with Rhizopus oligosporus. This compound has excellent antioxidant activity and may explain the stability of oil in tempeh reported by Murakami et al. (54).

Recently, daidzein, genistein, glycitein and their glycosides were measured in American and Japanese soybeans, and commercial soybean foods (17, 55). Data from these studies indicate that products such as soybeans, soy flour, soy protein and soy beverage powder have a total isoflavone content greater than 1 mg per g dry weight. In these soy products genistein and its derivatives are the most prominent isoflavones followed closely by daidzein (metabolized to equol). The total isoflavone content of such soy foods as miso, tofu and bean paste ranged from 0.3 to 0.6 mg/g whereas second generation soy foods (tempeh burger, tofu yogurt) had isoflavone levels in the range of 0.1 to 0.3 mg/g. Year and varietal variation of the isoflavone content of American and Japanese soybeans grown in Iowa also has been investigated (55). Substantial variation due to year (2-fold), American variety (2-fold) and growing location (50% difference) was observed. In general, Japanese varieties of soybeans had higher levels of total isoflavones than American varieties.

These observations demonstrate that soybeans and soy products are major sources of isoflavones. However, only a limited number of retail soy-based foods have been analyzed for isoflavones. In addition, other legumes (peas, green beans and lima beans) generally are more highly consumed in the U.S. than soy-based foods, yet there is a dearth of isoflavone data for these foods.

E.2.7. Methods for phytoestrogen (isoflavone) analysis. Naim et al. developed a gas chromatographic method for separation and quantitation of the trimethylsilyl derivatives of genistein and diadzein (33). However, over the last decade, high performance liquid chromatography (HPLC) has become the method of choice for the measurement of isoflavones in foods and food products. Murphy measured genistin and daidzin and their aglycones as well as a minor component, coumestrol, in soybeans and soy-foods with a reversed-phase column and ultraviolet detection system (56). This method reported extraction efficiencies and recovery data that had not been reported previously. The level of detection in this system was about 8 ppm genistin (-ein) and 12 ppm daidzin (-ein). Eldridge developed a similar procedure for measuring the common isoflavones as well as glycitin (57). Setchell, et al., developed a sensitive isoflavone analysis system by combining a reversed-phase column system with an electrochemical detector (45). These workers also interfaced the outlet of the HPLC column to a mass spectrometer which greatly aided in the identification of isoflavones that were separated on the HPLC column. Recently, Murphy's group developed a reversed-phase HPLC system employing a photodiode array detector (PDA) that is capable of separating daidzein, genistein, glycitein, their glucosides and derivatized glucosides in about 60 minutes (17). A total of twelve isoflavones and derivatives were separated and quantified in soybeans and soybean food products. Absorption spectra from the PDA as well as retention times derived from pure standards were used to identify chromatographic peaks.

Farmakalidis and Murphy reported a semipreparative HPLC method for the isolation and purification of isoflavonoid glucosides (51). This method greatly reduced the time required to isolate milligram quantities of isoflavonoids.

From this brief review, it is apparent that technology and techniques are available for the separation and quantification of isoflavones in foods and food products. Dr. P.A. Murphy and her associates have been instrumental in the development and application of many of these methods. As a collaborator of the proposed research, her laboratory has appropriate instrumentation and expertise

to successfully complete the analytical portion of the project.

E.2.8 Tabulated data on the phytoestrogen (isoflavone) content of foods. Considerable data exist on the qualitative aspects of isoflavones in foods. However, only limited analytical data are available. Eldridge measured the concentration of five isoflavones in commercial soybean flours, concentrates and isolates (44). Murphy evaluated the level of several prominent isoflavones in a wide variety of soy-protein products (42). More recently this group measured the level of twelve isoflavones in several soy ingredients as well as traditional and second-generation soy foods (17). These scientists also evaluated the effect of crop year and soybean variety on isoflavone content (55). Several additional publications report the isoflavone content of soy-products on a limited basis (56, 57).

Foods used in these analysis studies were purchased or derived from limited sources (single stores or processed from a single batch of soybeans). Data on the content and variability of isoflavone levels of foods purchased from retail stores in several large metropolitan areas is unavailable. The lack of a comprehensive tabulation of prominent isoflavone species in the US food supply that may have "anti-estrogenic" activity greatly impedes the ability of the epidemiology and cancer research community to elucidate the role of this group of food components in the reduction of breast cancer risk.

E.2.9. Necessity for a food phytoestrogen (isoflavone) database. Epidemiologic studies that investigate diet-health relationships associate food intake with health and status and disease incidence. The association of specific food component(s) with the health (disease) state of individuals requires detailed databases of food composition. Traditionally, the United States Department of Agriculture has maintained food composition databases that contain the nutrient content of foods commonly consumed in the U.S. (58). However, as epidemiologic studies expand the number of possible food components that may reduce cancer risk (59), traditional databases are unable to "keep pace" with demand for data on "new" food components. As a result, collaborations of experts have formed to develop databases for a specific nutrient or family of food components. Examples of these databases include selenium (60) and carotenoids (61, 62).

Considerable information relative to the specific role that phytoestrogens (isoflavones) may play in reducing the risk of breast cancer can be ascertained from existing epidemiologic data if a valid food phytoestrogen database is made available. In addition, the availability of a food phytoestrogen database will permit the design of new epidemiologic and controlled feeding studies to further elucidate the role of "phytoestrogen-rich" foods in the reduction of breast cancer risk. To be most useful, this database should have representative values for each of the isoflavones and their various forms (aglycone, glucoside) for those foods that supply the majority of these components in the diets of those sub-populations surveyed by epidemiologic studies (U.S.). In addition, data are needed for the isoflavone content of soybean preparations that are major ingredients of processed foods. This Infrastructure Enhancement proposal will provide a first generation food phytoestrogen database which can be used by the greater diet-health community to assess the importance of these dietary components in reducing the risk of breast cancer.

E.3. Purpose

Develop a database containing concentrations of specific isoflavones for soybean-based food products and other legume foods commonly consumed by human beings in the U.S.

E.4. Technical Objectives

- Aim 1. Measure isoflavone content of selected soybean products, soybean based foods and other isoflavone-containing food products which are available at retail stores.
- Aim 2. Ascertain the influence of cooking and processing on levels of isoflavones in selected foods and products.
- Aim 3. Evaluate, tabulate and summarize isoflavone content of legume foods.
- Aim 4. Calculate isoflavone intake of selected sub-populations in the U.S. This activity will demonstrate one application of the database.

E.5. Methods

E.5.1. Selection of soy-products and foods. Several forms of soybeans are used in the human diet, as unique foods such as tofu, miso, soymilk, soynuts, tempeh, and as ingredients in other foods including soups, bakery products and other prepared foods. As minor ingredients in some foods, soy isolates and concentrates, meal, and flour are used to increase water holding capacity, to improve texture and other sensory properties, and to contribute to the protein content of the food. From available data, over 90% of soy-products consumed by human beings in the U.S. are consumed in the form of soy isolates or concentrates that have been incorporated into foods. Tofu and soy sauce constitute less than 5% each and such foods as miso, soynuts, tempeh and soy milk beverages total about 2%.

Based on the above information, foods will be selected that represent each category of soy products. The list of soy products and foods proposed to be sampled is tabulated in Tables 1 and 2. Selection of specific brands will be based on sales volume, whenever possible. These decisions will be made during the preparation of sampling plans during the first year of the study (Figure 2). Two products, soy/beef hamburgers and tofu, will be analyzed both "raw" and cooked which will provide estimates of the stability of the isoflavones to the cooking process. Products that are normally consumed cooked, i.e., miso, vegetable protein products, will be prepared according to package directions.

E.5.2. Sampling of soy-products and foods. A broad-based nationwide sampling plan that incorporates statistically based sampling techniques and pertinent demographic information will be used to develop a multistage cluster sampling plan (63). For the sampling of soy-based foods, the country has been divided into five geographic regions (Northeast, Southeast, North Central, Southwest, and West). Within each region, one or two cities ranking highest in supermarket sales have been identified (New York, Washington-Baltimore, Tampa, Kansas City, Houston, and Los Angeles). Due to the nature of the consumption of soy-based foods, two cities that have large vegetarian populations will also be sampled (Boulder, CO and Cedar Springs, MI). Los Angeles is unique in that it ranks first in supermarket sales and also has a large vegetarian population. As a result, foods that are available in retail stores, will be purchased in eight cities which represent major population centers of the United States, regions with high densities of vegetarians and geographic (food product) variability.

The leading supermarket chains or health food stores (2-5 chains) will be chosen within each city

Table 1. Sampling strategy for foods for isoflavone analysis

Food Product	Scope of Sampling	Type of Store	Comments	Number of Analytical Samples
Alfalfa sprouts	Eight cities	Grocery chain	Example of non-soy food	8
Infant formula	Eight cities	Grocery chain	Brands selected on sales volume	8
Miso	Eight cities	Health food	Example of fermented food	8
Soy/beef hamburgers raw and cooked	Military procurement	--	Two or three major formulations; sampled during three seasons	18
Soy concentrates	Major producers: Ralston Purina Archer Daniels Midland One additional producer	Direct from producers	Concentrates selected based on production for human food manufacturers; sampled during three years	9
Soy isolates	Major producers: Ralston Purina Archer Daniels Midland One additional producer	Direct from producers	Isolates selected based on production for human food manufacturers; sampled during three years	9
Soymilk Edensoy +2 addn'l brands + 2 dry brands	Eight cities	SDA ¹ Health food	Brands selected on sales volume; sampled during seasons	8
Soy oil 3 brands	Eight cities	Grocery chain	Brands selected on sales volume	8
Soy sauce 2-3 brands Fermented vs. non-fermented	Eight cities	Grocery chain	Brands selected on sales volume; fermented and non-fermented analyzed separately	16
Tempeh	Eight cities	Health food Ethnic food	Brands selected on sales volume	8
Tofu 3 brands Raw and cooked	Eight cities	Grocery chain Health food Ethnic food	Brands selected on sales volume; coagulants considered. Raw and cooked composites analyzed separately	16
Vegetable protein product "Chicken" analog "Vege" link "Meat" patty	Eight cities	SDA Major outlets for soy-based analogs	Final product selection based on sales volume	24

¹Stores that specialize in food products for Seventh Day Adventists.

Table 2. Additional foods for isoflavone analysis

Source of Food	Foods and Scope of Sampling	Comments	Number of Analytical Samples
Storage (-50°C) stability study	Tofu from a homogeneous batch stored in ~ 100g aliquots; Analysis conducted at 60 day intervals	Products prepared in pilot plant at ISU	7
Japan	Miso, roasted soybeans, soymilk, tempeh and tofu; sampled in Tokyo, Japan	Foods purchased in Asahi Foods store. One composite for each food	6
Soybeans grown in Iowa	Three varieties, three crop years, raw soybeans and three products	Products prepared in pilot plant at ISU	36

to account for approximately 50% or more of the grocery sales (64). As an example, in the Washington-Baltimore area, two supermarket chains (Giant and Safeway) account for about 70% of the area's grocery sales and will be the chains sampled in this study. Health food and ethnic food stores will be selected in a similar manner. Final sampling plans, in terms of number of stores/samples, will be based on variance estimates calculated from analysis of phytoestrogens in foods during a preliminary limited nationwide sampling (two or three cities). In general, one or two stores of each type (grocery, health, ethnic, etc.) will be randomly selected (telephone books) and representative foods will be purchased during periods of average to high product turnover (late-week or weekends). In the case of soy/beef hamburgers, soy isolates, and soy concentrates, foods will be procured directly from suppliers.

A contract with a food sampling service (Superior Products, Inc., Chicago, IL) will be negotiated for the procurement, packing and shipping of all samples from metropolitan areas to Dr. Murphy's laboratory at Iowa State University. Detailed instructions will be provided to the contractor relative to precise store location(s), detailed description of food including brand name(s) and package size, method of packing and method of shipment (ambient temperature, "blue ice" or dry ice). All samples will be shipped to Iowa State University via overnight delivery.

At the laboratory, foods will be given an identification number and logged into a sample management system. Subsequently, all retail foods will be prepared as normally consumed following label directions. Equal weights of each sample for a specific food, (e.g., tofu) within a city will be composited. As a result, each food product that is sampled in metropolitan areas will result in eight samples for analysis (one sample for each city, see Table 1). All foods will be comminuted in a food processor, moisture analysis will be conducted and aliquots of each sample will be frozen and stored (-50°C or lower) for subsequent analysis. During the first twelve months of the project, studies will be conducted to validate the stability of each common isoflavone and its derivative in frozen samples.

Selected soy-foods (miso, soymilk, soy sauce, tempeh, and tofu) also will be sampled in a large metropolitan area of Japan (Table 2). As an example, Asahi Foods stores will be one chain sampled in the Tokyo area. The same general sampling procedure as described for the United States will be applied to Japan. These studies will be conducted with the assistance of Dr. K. Yasumoto, Research Institute for Food Science, Kyoto University, Kyoto, Japan.

Selected varieties of soybeans that are major sources of human foods in the U.S. also will be sampled and analyzed. A total of seven varieties will be evaluated including Vinton 81, Prize and Enrei, the preferred Japanese tofu variety. All soybeans will be grown under defined conditions and provided by Dr. C. Hurburgh, Department of Agricultural and Biosystems Engineering, Iowa State University and Dr. R.G. Palmer, Department of Agronomy, Iowa State University. These varieties will be sampled during three crop years. Preserved identity soybeans from a single variety will be processed into soy flakes, soy isolates (from soy flakes), soy concentrates, extruded soy protein (textured vegetable protein), soy sprouts and fermented soy products, miso, natto and soy sauce. Mass balance and isoflavone isomer distribution will be determined. Data from these studies will provide an estimate of the year-to-year variability of the isoflavone content in soybeans and soy-products grown and processed in Iowa.

E.5.3. Measure isoflavones in foods.

E.5.3.1. Instrumentation. The high performance liquid chromatography (HPLC) apparatus will consist of a Beckman-Altex Model 420 microprocessor system controller, two Beckman-Altex Model 110A liquid chromatograph pumps, Beckman mixer, a Waters 991 Photodiode Array Detector (PDA) monitoring 200-350 nm, and a NEC computer with Waters PDA data processing software (Budget request). A YMC-pack ODS-AM-303 column (5 μ m, 25 cm x 4.6 mm i.d.) will be used for quantitative HPLC analyses. A YMC-pack ODS-AM-323 column (10 μ m, 25 cm x 10 mm i.d.) will be used for semipreparative HPLC. All HPLC analyses will be performed at ambient temperatures.

E.5.3.2. Isoflavone standards. Ten isoflavone standards will be used for quantifying the amounts of isoflavones in the food samples. Authentic standards of daidzein, genistein and coumestrol will be obtained from commercial sources (ICN Pharmaceuticals, Plainview, NY, and Calbiochem Corporation, La Jolla, CA). Daidzin and genistin will come from previous work in the laboratory. 6"-O-Acetyldaidzin and 6"-O-acetylgenistin will be isolated according to the method of Farmakalidis and Murphy, except that semipreparative HPLC, instead of crystallization, will be used for the final purification. A linear gradient of 0% to 60% of acetonitrile (ACN) in 30 min at a flow rate of 2 ml/min will be used to separate 6"-O-Malonyldaidzin from daidzin and 6"-O-malonylgenistin, and 6"-O-malonylglycitin will be purified as follows: ground soybeans will be defatted with hexane for 1 h at room temperature. Defatted flour is extracted with ACN and 0.1 N NaCl (1:5:1 w:v:v) according to Murphy for at least 2 h at room temperature, then filtered through Whatman No. 42 filter paper. The filtrate is concentrated by using a rotary evaporator at 30°C. An aliquot is applied to 2.5 x 75 cm Sephadex-LH 20 column with eluent of 50% methanol (MeOH) to separate 6"-O-malonylgenistin from 6"-O-malonyldaidzin and 6"-O-malonylglycitin. Further purification to 6"-O-malonylgenistin is achieved with a semipreparative column and isocratic mobile phase of 25% ACN with 0.1% glacial acetic acid. 6"-O-Malonyldaidzin and 6"-O-malonylglycitin are separated from each other employing semipreparative HPLC and a mobile phase of 10% ACN and 0.1% glacial acetic acid. Confirmation of identity will be based on data from Kudou et al. (65). The malonylisoflavones in crude extracts degrade into glucosides after 5 days, even when stored at 5°C. During extraction, acidic conditions and temperatures lower than 30°C are necessary for preventing malonylisoflavones from degrading. Glycitin is purified according to the method of Naim et al. with a slight modification. Pure 6"-O-acetylglycitin and glycitein have not been obtained because of their minute amounts in soybeans from Iowa. The quantitation of these two compounds in the soy-foods is estimated by using the same standard curve of glycitin and by adjusting for the molecular weight differences. Coumestrol will be quantified when present and is expected in germinated soybean products and alfalfa sprouts.

E.5.3.3. Isoflavone extraction. Two g of freeze-dried, finely ground samples are placed in a 125 ml screw-top Erlenmeyer flask containing 10 ml of ACN and 2 ml of 0.1 N HCL and stirred for 2 h at room temperature. Extractants are filtered through Whatman No. 42 filter paper. The filtrate is taken to dryness on a rotatory evaporator at $\leq 30^{\circ}\text{C}$. The dried material is redissolved in 10 ml of 80% HPLC-grade MeOH in water. A aliquot of the sample is filtered through a 0.45 μ m PTFE filter unit (polytetrafluoroethylene, Alltech Associates, Deerfield, IL) and analyzed by HPLC. Moisture content of the sample is determined according to the AOAC method. Recovery estimates are evaluated by adding genistein standard to dry samples and extraction with solvents. Recovery data of five soy-foods have been as follows: textured vegetable protein, 70% ($\pm 16\%$); soy beverage, 79% ($\pm 13\%$); tofu, 95% ($\pm 5\%$); "fakin' bacon", 90% ($\pm 11\%$); and tempeh, 86% ($\pm 7\%$).

E.5.3.4. HPLC quantitation of isoflavones. A linear HPLC gradient is composed of (A) 0.1%

glacial acetic acid in H₂O and (B) 0.1% glacial acetic acid in ACN. Following injection of 20 µL of sample, solvent B is increased from 15% to 35% over 50 min, then held at 35% for 10 min. The solvent flow rate is 1 ml/min. A Waters 991 series Photodiode Array Detector is monitored from 200 nm to 350 nm. The minimum detectable concentrations for daidzein and genistein are 185 and 100 ng/ml, respectively. UV spectra are recorded and area responses are integrated by Waters PDA software. An HPLC pulsed amphoteric detector is also available for analysis of extracts that have very low levels of isoflavones. All extracts will be analyzed in triplicate; outlier values will be discarded and means of remaining data calculated.

E.5.3.5. Analytical Quality Control. Several steps will be taken to assure that the extraction, separation and quantitation procedures are adequate and validated before foods are analyzed. These include: 1) Recovery of spikes - Recovery of pure isoflavones added to representative foods (Vinton 81 soybeans, Een soymilk, tofu, tempeh and TVP) during the grinding step and prior to extraction with isoflavonoid solvents. Recoveries of 92-102% will be considered acceptable. Extensively heat-treated soy products (TVP) may cause some problems. 2) Within day precision - Extraction and analysis of 4 aliquots of the same homogeneous food for isoflavones on the same day. A coefficient of variation of 6% or less for each isoflavone will be considered acceptable. 3) Between day variation - The procedures outlined in 2) above will be conducted over 5 days. A coefficient of variation of 8% or less for between day error for each isoflavone will be considered acceptable. 4) Analysis of reference materials - Large batches of two foods (Vinton 81 soybeans and Eden soymilk) will be obtained and stored at -60°C for the duration of this project and analyzed periodically.

During the analysis of foods, two additional quality control steps will be conducted. These include: 1) Pure reference standard analysis - Mixtures of pure reference standards will be analyzed each day samples are analyzed. Peak areas for each isoflavonoid standard will be plotted and theoretical concentrations calculated from standard curves. Deviation from 92-102% recovery or negative positive trends will indicate analytical problems necessitating correction and standardization before continuing with food analysis. 2) Reference material analysis - The two reference materials listed in 4) above will be analyzed periodically (twice a month) and the resulting data plotted. Any severe divergence from the mean value ($\pm 10\%$) or negative/positive trends will indicate that the measurement system must be corrected and revalidated. We do not intent to use a recovery of internal standard method because of concerns about an appropriate chemical as the internal standard. Eldridge used an internal standard for his analysis of isoflavones (44). We are not convinced that we can find an appropriate internal standard that will mimic the behavior of soy isoflavones adequately for our purposes.

E.5.3.6. Statistical analysis. Where appropriate (varietal differences, etc.) statistical analysis will employ the procedures developed by the SAS Institute, Inc. (Box 8000, Cary, North Carolina). Analyses of variance using the general linear models (GLM) will be conducted, and differences between the sample means will be analyzed by Fisher's Least Significant Difference (LSD) test.

E.5.4. Evaluate and tabulate isoflavone content of foods. The impact of food components on health can be ascertained only with data that are accurate, precise and representative of foods in the food chain. Mangels et al. (61) recently described an artificial intelligence system for the evaluation of analytical data of the carotenoid content of foods. The product of this system is a table containing food aggregate names and estimates of central tendency (median), range of values and a level of confidence for the five most important dietary carotenoids. We propose to modify this system for the critical evaluation and tabulation of prominent isoflavones in foods. A brief

description of the process follows.

E.5.4.1. Develop data evaluation system. For carotenoids, analytical data were evaluated in five general categories: analytical method, analytical quality control, number of samples, sample handling and sampling plan. These categories represent the major determinants of data quality for inclusion in a food composition table or database and are expected to be the same for isoflavone data. Specific criteria will be developed for each category with a rating for each ranging from 0 (unacceptable) to 3 (highly acceptable). Criteria for both analytical method and analytical quality control will be developed to address the acceptability of methodology for isoflavone analysis and will follow the general procedures outlined by Mangels et al. (61). Similarly, criteria for the other categories will be developed or adapted specifically for isoflavones in foods. After the criteria are established, decision trees or pathways will be developed and the existing artificial intelligence software for carotenoids will be modified.

E.5.4.2. Data compilation and evaluation. The scientific literature will be extensively reviewed. Only those studies which utilized a chromatographic procedure to generate data will be evaluated. Data for only the most prominent isoflavones in foods will be evaluated. Although it is difficult to identify the most prominent isoflavones without extensive literature review, it is anticipated the following are likely candidates: Daidzein, genistein, and their glucosides. The total isoflavone content will be tabulated (aglycone plus glycoside) since the metabolic fate of flavonoid glycosides is unknown in human beings. It is anticipated several calculations will need to be performed on published data. For example, values expressed as the carbohydrate derivative of an isoflavonoid will be converted to the amount of the aglycone form of the isoflavone present using appropriate ratios of molecular weights. Values expressed on a dry weight basis with accompanying moisture values will be converted to a wt weight basis prior to compilation.

During the data evaluation process, the database nutritionist will rate each isoflavone value for a food in a specific reference by answering questions in each category posed by the artificial Intelligence system. Next, a Quality Index, an indicator of the overall data quality for a flavonoid value in a food from a single study, will be calculated. In general, the average of ratings for the five general categories is the Quality Index. A zero rating for analytical method or for any three ratings will result in a Quality Index of zero for that publication. A Quality Index of one or more will be used as the level for an acceptable value.

Similar foods will be grouped into aggregates in order to reduce the number of entries in the food table and increase the validity of data. If after evaluation of data, there is a clear stratification by distinct forms of the food, then the forms of that food will be separated into two or more aggregates. In general, the final aggregation of data will be based on approximate similarity of food descriptions within an aggregate.

E.5.4.3. Development of tables for individual isoflavones in foods. Reported values will be used to calculate the central tendency (mean or median, determined from evaluation of data distribution) of each isoflavone for a food aggregate with equal weighting of each value. All means or medians will be rounded appropriately based on the analytical detection limit and the least number of significant digits in a contributing value.

Variability of data will be expressed as standard deviation or minimum/maximum values depending on the indicator of central tendency and number of observations. In addition, the number and citations of references that yielded acceptable data will be documented. An indicator of the quality

of each mean or median (confidence code) will be determined based on the sum of the Quality Indices for that food aggregate (61).

The product of this research will be a food isoflavone database that will be made available to the diet-health community at no cost. These data will provide the basis for ascertaining the role of food isoflavones in the reduction of breast cancer risk. Isoflavone data tables will be compiled and published in peer reviewed journals. These data also will be transferred to the Nutrient Data Research Branch, HNIS, USDA for inclusion into USDA Handbook No. 8 and the Nutrient Data Bulletin Board.

E.5.5. Calculate isoflavone intake of selected subpopulations in the U.S. The methods for the calculation of isoflavone intake in populations and sub-populations will follow the procedures used by Chug-Ahuja et al. for the determination of carotenoid intake in adult women (62). Briefly, the procedures will consist of linking the database of isoflavone values for individual foods (developed as part of this project) with the U.S. Department of Agriculture Survey Nutrient Data Base recipe file and subsequently develop a database for individual isoflavones in soy-foods, other legumes and multicomponent foods containing soy-products and soy-foods. This database not only will consist of food values for individual isoflavones but also each food will be coded to the food codes used in the U.S. National food and health surveys (National Food Consumption Survey and National Health and Nutrition Examination Survey). These codes will permit easy linkage of the isoflavone database to the large surveys that collect food intake data as well as most other smaller surveys.

Subsequently, the complete isoflavone database will be linked to specific food intake surveys and the intake of individual as well as total isoflavones will be calculated. In addition, results from these calculations will be used to identify those foods that are the major sources of isoflavones in the diet. Food intake surveys that will be used to estimate isoflavone intake include 1991-93 NHANES (Phase I), 1994 National Food Consumption Survey (pending availability of data). In addition, selected surveys also will be used that have reported food intakes of sub-populations that are known to have reduced incidence of breast cancer. These include vegetarians, Seventh Day Adventists as well as others. For these studies, collaboration will be established with the principal investigator for each survey to assure appropriateness of the survey, integrity and completeness of food intake data and reasonableness of outcome results.

F. Body of Report

F.1. Aim 1. Measure isoflavone content of selected soybean products, soybean based foods and other isoflavone-containing food products which are available at retail stores.

F.1.1. Selection of soy-products and foods. The specific foods which have/will be sampled for subsequent analysis are tabulated in Tables 1 and 2. No significant changes are expected in this list which was submitted as part of the original grant proposal.

F.1.2. Sampling of soy-products and foods. To date, soy-based infant formula, soy concentrates, soy isolates (Table 1) several varieties of soybeans grown in Iowa and foods from Japan (Table 2) have been sampled. With regard to soy-based infant formula, cans of formula from individual lots of the major manufacturers (Carnation, Gerber, Mead-Johnson, Ross, Wyeth) were purchased in grocery chain-stores in the following cities: Ames, IA, Beltsville, MD, Chicago, IL, Los Angeles, CA and San Francisco, CA. The selection of the grocery chain was based on the largest market share for their respective region; individual stores were selected randomly. A contractor was employed to select, procure and ship, under rigid guidelines, products in Chicago, San Francisco and Los Angeles. Samples from the other cities were selected and purchased by members of the research team. All available brands of soy-based formula from each manufacturer (two brands from Mead-Johnson, one brand from all other manufacturers) were selected. Soy concentrates and soy isolates from several production lots of the major manufacturers of these products (Archer-Daniels Midland, Central Soya, Protein Technologies International) were provided by the manufacturers. Representative aliquots of beans have been sampled from test plots at Iowa State University of several varieties of soybeans typically grown by Iowa farmers. Several soy-based products (thin fried tofu, thick fried tofu, fermented soybeans [nattou], soybean flour [kinako], and a by-product of tofu production [okara] were purchased in one of the large grocery stores in Tokyo, Japan by a US scientist attending a scientific conference. All samples were shipped for subsequent analysis of isoflavones to the laboratory of Dr. P. Murphy, co-investigator of this grant, at Iowa State University under conditions that retained sample integrity (ambient for canned products, dry ice for other products).

Relative to the remainder of the soy-based foods tabulated in Table 1, sampling plans currently are being developed. Market share information for brand names based on grocery store scanning data have been ordered from Nielsen North America for soy sauce, canned soy milk, powdered soy milk, soy oil and soy based meat analogs (vegetable protein product in Table 1). Generally, these products are processed and produced in central manufacturing facilities. Based on high market share data, brands names will be identified for subsequent sampling in New York, Baltimore-Washington, Tampa, Kansas City, Houston, Los Angeles, San Francisco and Boulder, CO. Alfalfa sprouts, miso, tempeh and tofu are manufactured both centrally and locally. As a result, at this time we are attempting to identify major brand names for sampling in grocery chain stores in each of the above cities. In addition we have identified colleagues who are willing to help and are knowledgeable about the dietary habits and supply of these products in the Chinese section of San Francisco and Japanese section of Los Angeles. With their assistance, we will also sample these four products from stores in the ethnic sections of San Francisco and Los Angeles. Simultaneously, guidelines are being developed for detailed procurement, packaging and shipping

of food samples by the contractor (Superior Products Inc.). Finally, we are in the process of making contact with appropriate officials in the military food procurement offices to assist us in the acquisition of soy/beef hamburgers typically supplied for consumption by military personnel (Table 1). It is anticipated that sampling details for the remainder of the products will be completed by May 1, 1996.

F.1.3. Measure isoflavones in foods. A new high performance liquid chromatography system (HPLC) was purchased, installed and validated. The system includes pumps, injector, column (YMC-ODS-AM-303) photodiode array detector and PC system controller/data manager. The analytical HPLC run was modified and shortened from 90 min to 60 min to accommodate increased daily throughput. Part of this modification was to increase column temperature to 30 degrees C to avoid retention time drift due to room temperature fluctuation. Standards for each of the isoflavones and their derivatives have been isolated from soybeans, purchased or synthesized. Standard curves have been prepared both for quantification of isoflavones in food samples and for the development of spectral libraries of each isoflavone to validate identification in extracts of foods. An intermediate in the synthesis of genistein and daidzein has been identified as a viable internal standard both for anchoring chromatographic retention times and for validating the extraction and preparation of each sample.

Extraction procedures for the quantitative removal and isolation of isoflavones from various foods and products were modified to improve extraction efficiency and accuracy. For example, infant formulae and textured soy proteins require increased proportions of water in the extraction buffer to prevent the soy-containing materials from forming a gum-like mass. These modifications were validated by checking for maximum extraction of isoflavones from the sample and recovery of the internal standard.

The analytical quality control system for the measurement of isoflavones in soy-based foods and commodities has been established. This system consists of a software program that captures pertinent information for each sample that is received and generates a unique sample number and label for use throughout the subsequent analysis and data compilation. Backup diskettes and tapes are being used to archive sample information and data. Another important component of the quality control system is the in-house quality control (QC) sample program. Soybean flour and soymilk have been identified as appropriate QC materials. A large batch of each product from a single production lot was provided by a manufacturer of the respective products. Long term storage of each QC material at room temperature and at -70 degrees C has been underway since August 1995. Analysis of both products stored at both temperatures is conducted monthly. Data are immediately calculated and tabulated on a CUSUM plot to assess the validity of the analysis for each isoflavone and its derivatives. Within day sampling and replicate day sampling of these same products have been ongoing since September 1995. Quality assurance standards of solutions of selected pure isoflavones are run once per week to evaluate detector lamp performance and deviations. Minor deviations are used to adjust the slopes of standard curves for each isoflavone; major deviations indicate repair and service of the detector is required. Pure isoflavone standards also are run each day food samples are analyzed and the data used in the same way as discussed above.

All sampled soy-based products and foods have been analyzed or are being analyzed currently.

Prior to drawing any definitive conclusions the data must be statistically analyzed following procedures outlined in section E.5.3.6. above. Preliminary perusal of data from the soybean varieties typically grown in Iowa suggest a wider variability in isoflavone levels among the crop grown during 1995 than that observed in the 1992 crop. Possible explanations for this change currently are being sought.

F.2. Aim 2. Ascertain the influence of cooking and processing on levels of isoflavones in selected foods and products.

A miso and soy sprout production system have been established at Iowa State University (ISU). Miso and soy sprouts have been produced with these systems and the mass balance distribution of isoflavones during production has been determined for each of these foods. A natto fermentation system is being developed at ISU from which similar data will be generated for this food. Retention of isoflavones in cooked foods, ie., soy/beef hamburgers, vegetable protein products (see Table 1), will be determined as part of the analysis of these foods after they have been sampled from retail grocery stores.

F.3. Aim 3. Evaluate, tabulate and summarize isoflavone content of legume foods.

The data evaluation system developed for the critical evaluation of the carotenoid content of fruits and vegetables (61) currently is being modified for the evaluation of isoflavone content of soy-based products and foods. To accomplish this modification, a predoctoral student in the Department of Computer Science at George Washington University (GWU) has been employed through a specific cooperative agreement between the Agricultural Research Service/USDA and GWU. In particular, the section of the system that evaluates analytical method, validation of method and analytical quality control must be modified to accommodate the uniqueness of the isoflavone procedures as compared to carotenoid analytical processes.

Literature references containing data on the isoflavone content of soy and soy-based food products are being identified and reviewed for validity and appropriateness. Isoflavone data in these publications will be entered into the data evaluation system as soon as the software has been modified and tested.

F.4. Aim 4. Calculate isoflavone intake of selected sub-populations in the U.S.

The start of activity on this Aim requires completion of research on Aims 1-3. The procedures required to complete Aim 4. have been detailed in section E.5.5. above.

G. Conclusions

The ultimate goal of this research is to provide scientific community with a database of values for the various isoflavones prominent in soy-based commodities, ingredients and foods. The critical research components for the successful completion of the goal are: 1) develop and validate rugged analytical procedures for the accurate and precise measurement of prominent isoflavones in appropriate foods and food components, 2) establish appropriate analytical quality control system which will monitor analytical accuracy and precision, monitor quality assurance of analyte identification during measurements and provide criteria for acceptance of analytical data, 3) identify commonly consumed and utilized soy-based foods, ingredients and commodities, 4) develop sampling schema for each soy-based product to assure that representative food samples are selected for subsequent analysis, 5) prepare foods for analysis as they normally would be consumed or used in food preparation, composite appropriate samples to obtain representative data, and analyze composites for isoflavone content, and 6) develop critical data evaluation and summarization system for the review of all published and non-published values pertaining to the isoflavone content of foods. The summary generated as part of this activity will be the database of isoflavone values.

Accomplishments to date as part of this grant include completion of the following critical research components: 1) develop and validate rugged analytical procedures, 2) establish appropriate analytical quality control system, and 3) identify commonly consumed and utilized soy-based foods, ingredients and commodities. Nationwide sampling schemes (research component 4) have been developed for several foods which have been selected and analyzed. For the remainder of the foods, pertinent data and information are being collected for the completion of the sampling schemes. Several foods and commodities have been prepared, composited and representative aliquots analyzed (research component 5). Analyses of foods are completed as soon as possible after samples are received. Relative to research component 6, the data evaluation system is in the process of being modified to accommodate isoflavone data. The isoflavone database will be generated as the final product of the data evaluation process.

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